THE CONSEQUENCES OF METHOXYCHLOR EXPOSURE ON THYROID HORMONE RESPONSE ELEMENT DR4 REGULATED GENE EXPRESSION

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Consequences of Methoxychlor Exposure on Thyroid Hormone Response Element DR4 Regulated Gene Expression. (May 2015)

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Neural development is initiated by several signaling molecules including BMP-4, noggin, and chordin. These molecules induce the formation of neural plate, which later folds to become the spinal cord and part of the primitive brain. Because these signaling axes are sensitive to external factors, the process of neural development is subjected to an intense level of vulnerability. An environmental toxin, such as methoxychlor, can disrupt the molecular signaling axis during development An insecticide used as a replacement for DDT, methoxychlor was removed from the market due to its ability to contaminate drinking water, resulting in reproductive problems. Methoxychlor's endocrine disrupting properties have warranted a closer look at its role, if any, in disrupting normal neural development.

Studies on transcription factors such as Sox2, which is responsible for maintaining neural stem cells in their pleuripotent state, have suggested that thyroid hormone has a significant role in the progression of neural development. A closer examination of genes containing thyroid hormone response elements can be utilized to gain an understanding of thyroid regulated gene expression. We built a construct containing two DR4 thyroid response elements linked to a minimal SV40 promoter. This regulatory region drives luciferase expression, which we are able to measure in a

laboratory reporter assay. The aim of this project is to determine, via a luciferase assay, the effect of methoxychlor exposure in thyroid hormone's control of transcriptional activity during neural development.

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NOMENCLATURE

BMP-4: bone morphogenic protein-4

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: dimethyl sulfoxide

DR4: thyroid response element with half sites containing direct repeats, gap of 4 nucleotides

FBS: fetal bovine serum

GFP: green florescence protein

HEK 293 cells: Human Embryonic Kidney 293 cells

HPTE: 2,2-bis(p-hydroxyphenyl)- 1,1,1-trichloroethane

MCL: maximum contaminant level

MXC: methoxychlor

Pen/Strep: penicillin/streptomycin

Renilla: renilla- luciferin 2-monooygenase

Sox2: SRY(sex determining region)-box 2 gene

SV40: Simian vacuolating virus 40

T_3: triiodothyronine

T_4: thyroxine

TRE: thyroid hormone response elements

TRa1: thyroid hormone receptor alpha-1

TRb1: thyroid hormone receptor beta-1

TRb2: thyroid hormone receptor beta-2

TSH: thyroid stimulating hormone

CHAPTER I

INTRODUCTION

Embryonic Brain Development

The process of neural development begins shortly after gastrulation and is initiated by the signaling molecules BMP-4, noggin, and chordin. These molecules induce the formation of the neural plate and folding of the neural tube. The expression and localization of key transcription factors then form a boundary, known as the isthmic organizer, subdividing the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain). Dependence on these intricate signaling systems for proper development subjects the brain to an intense level of vulnerability to environmental toxins. Teratogenic substances have the potential to interfere with the epigenetic programming of a developing brain and its multiple signaling axes, leading to physiological abnormalities or neurological deficits.

Thyroid hormone physiology and role in brain development

Thyroid hormone is a lipophilic peptide hormone with a significant role in metabolism and nervous system maturation. There are three major isoforms of the thyroid hormone receptor: TRa1, TRb1, and TRb2 (Lazar 1993). TRa1 and TRb1 are expressed in almost all tissues, while TRb2 is predominantly seen in the anterior pituitary gland, likely to facilitate negative feedback of thyroid stimulating hormone (TSH) release (Bradley *et al.* 1992). Thyroid hormone receptors bind to thyroid hormone response elements (TREs), which are generally thought to be located upstream from the gene's promoter region. The most responsive TREs have been found to be

those in which the half-sites are arranged as palindromes, direct repeats (DRs), and inverted palindromes (IPs) (Yen 2001). The fetal brain expresses the TRa1 receptor almost exclusively, as opposed to the TRb1 receptor in the adult brain. Studies on rats have shown that TRb1 mRNA expression is increased postnatally (Strain *et al.* 1990). Thyroid hormone is synthesized in both the mature T_3 and inactive T_4 forms. A major constituent of both forms is iodine (T_3 and T_4 each contain three and four atoms of iodine per molecule, respectively). Intracellular deiodinases convert the inactive T_4 to the active T_3 form (Braverman *et al.* 1970). Studies have shown that hypothyroidism in pregnant women has the capacity to cause their child's poor performance on standard neuropsychological tests (as compared with children of matched control women). Neurological deficits were evident even in children whose mothers had experienced mild or asymptomatic hypothyroidism (Haddow *et al*. 1999). The fetus has the capacity to protect itself against maternal hypothyroidism by self-production of thyroid hormone. T_4 and T_3 production is observable by the 12^{th} and 30^{th} gestational weeks, respectively. However, a significant portion of brain development occurs earlier than these milestones, so neurological defects due to maternal hypothyroidism continue to exist (Zoeller 2003).

Because of the iodine component of both forms of thyroid hormone, hypoiodism during pregnancy can result in several physical and neurological defects, such as cretinism. Cretinism is characterized by stunted growth, severe developmental delays, neuromuscular deficits, and abnormal physical features. Maternal iodine deficiency that is left untreated, particularly throughout the first and second trimesters of pregnancy, has also been associated with neurological abnormalities, decreased head circumference, and a below average developmental quotient (Cao *et al.* 1994). Because thyroid hormone is crucial in the early development of the

brain, hypoiodism, and resulting hypothyroidism, has a significant impact on the proliferation of neuronal cells in several areas of the brain, including those affected by cretinism (particularly the cerebral cortex, cochlea, and basal ganglia). Potentially teratogenic effects of environmental toxins on iodine and thyroid hormone levels during early pregnancy represent a field of study that has yet to be thoroughly investigated.

Neural stem cells

Neural stem cells are characterized by their capacity to self-replicate and differentiate into neural progenitor cells. Neural progenitor cells subsequently give rise to mature neurons and glial cells. The subventricular zone (SVZ) is a paired structure on the lateral sides of the lateral ventricles of the developing brain (Doetsch *et al.*1997). As one of two sites of neurogenesis in both the pre- and post-natal brain, it contains a large population of neural stem cells.

Molecular role of thyroid hormone in neural stem cell development

Sox2 is a transcription factor implicated in maintaining a cell in its progenitor state. Repression of Sox2 is required for expression of factors favoring progression into mature neural cells. The enhancer region (upstream of the Sox2 promoter) contains three TREs. Increased levels of neural progenitor markers are correlated with decreased levels of expression of the TRa1 gene. In addition, increased expression of TRa1 shows down-regulation of the Sox2 gene's expression (Lopez-Juarez *et al.* 2012).

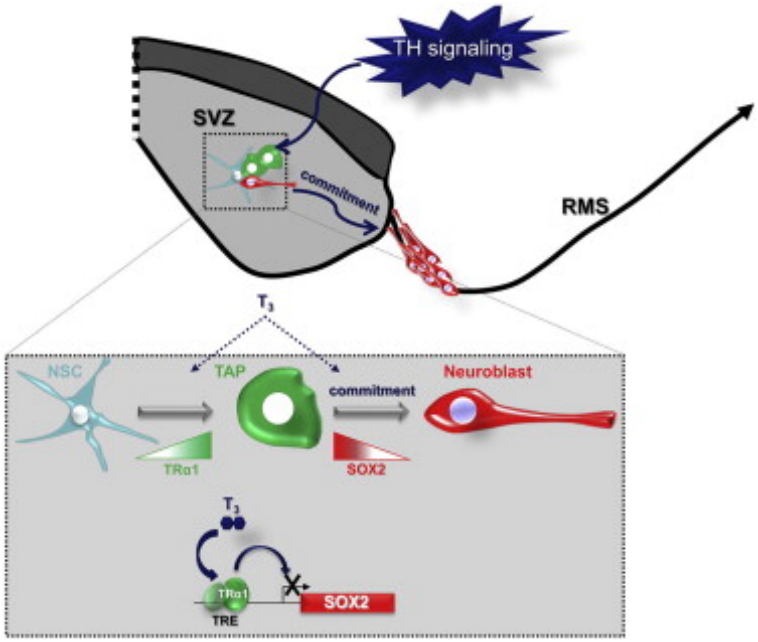

Figure 1: Proposed mechanism of impact of the thyroid hormone signaling axis on downstream Sox2 promoter (Lopez-Juarez *et al.* 2012).

Studies of the SVZ, which contains a heterogeneous population comprised of neural stem cells and migrating neuroblasts, have shown that the proportion of migratory neural cells increases (as the proportion of NSC's decreases) with the expression of the TRa1 receptor (Lopez-Juarez *et al.* 2012)

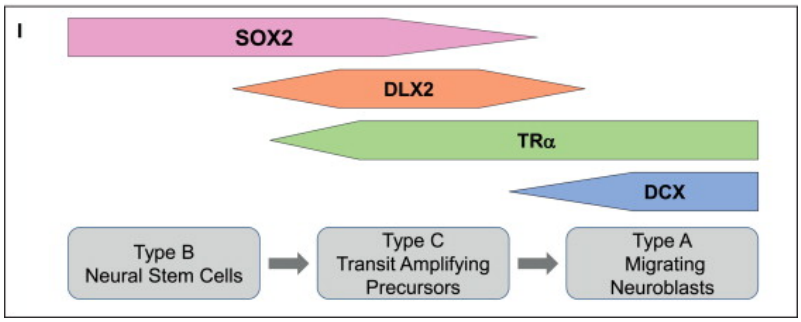

Figure 2: Summary of Sox2 and thyroid hormone receptor expression during neural stem cell commitment and migration (Lopez-Juarez *et al.* 2012)

Because thyroid hormone is required for nervous system development, the repression of Sox2 expression by thyroid hormone during stem cell differentiation has been proposed as a mechanism by which neurogenesis occurs.

Endocrine Disrupting Compounds

The term 'endocrine disrupting chemical' refers to a compound that alters hormonal systems and therefore endocrine endpoints. Most have a structural similarity to endogenous hormones and can interact with hormone receptors, resulting in the induction of epigenetic changes. A fetus *in utero* is especially vulnerable to changes induced by endocrine disrupting compounds due to a brief time period where epigenetic reprogramming is taking place (Fleisch *et all.* 2012).

Methoxychlor

Methoxychlor is a polychlorinated organocompound with a wide variety of physiological repercussions. It was originally used an insecticide that was used for insect and larval control as a replacement for DDT. It was banned from use due to its ability to contaminate drinking water through runoff and leaching from insecticide used on crops. The Environmental Protection Agency's (EPA) regulated Maximum Contaminant Level (MCL) is 0.04 mg/L. According to the EPA, long-term exposure to levels above the MCL can cause reproductive problems (Environmental Protection Agency 2009).

Endocrine disrupting activity of methoxychlor

The HPTE metabolite of methoxychlor is the teratogenic form of the pesticide. By mimicking estrogen, it has been shown to disrupt normal epigenetic programming, having a negative effect

on fertility and development in females (Cummings 1997). Methoxychlor has been shown to have adverse consequences in male reproduction as well. Studies in male rats have shown that testosterone production in Leydig cells is suppressed as a result of the HPTE metabolite (as well as other estrogenic insecticides), contributing to male infertility (Akingbemi *et al.* 2000). Because Leydig cells' production of testosterone is subject to inhibition by estrogen, decreased testosterone production upon exposure to methoxychlor demonstrates its endocrine disrupting activities.

Significance

While SOX2 has been the focus of several studies, this project will be examining the activity within promoter regions containing thyroid response elements. The construct used in this study has a DR4 TRE site linked to SV40 promoter, which drives luciferase expression. The aim of this project is to determine, via a luciferase assay, the effect of exposure to methoxychlor on gene transcriptional activity in embryonic and stem cell lines.

CHAPTER II

METHODS

Cell culture and media

HEK293 cells (ATCC Cat #CRL 1573) were maintained in DMEM (Invitrogen Cat #11965-092) supplemented with1% Pen/Strep and 10% FBS.

Transfection and treatment

Previously generated constructs were used in the transfection of HEK293 cells. The main reporter vector construct consisted of a DR4 site upstream of an SV40 promoter driving the Luciferase reporter gene, with a synthetic TRa1 thyroid hormone receptor.

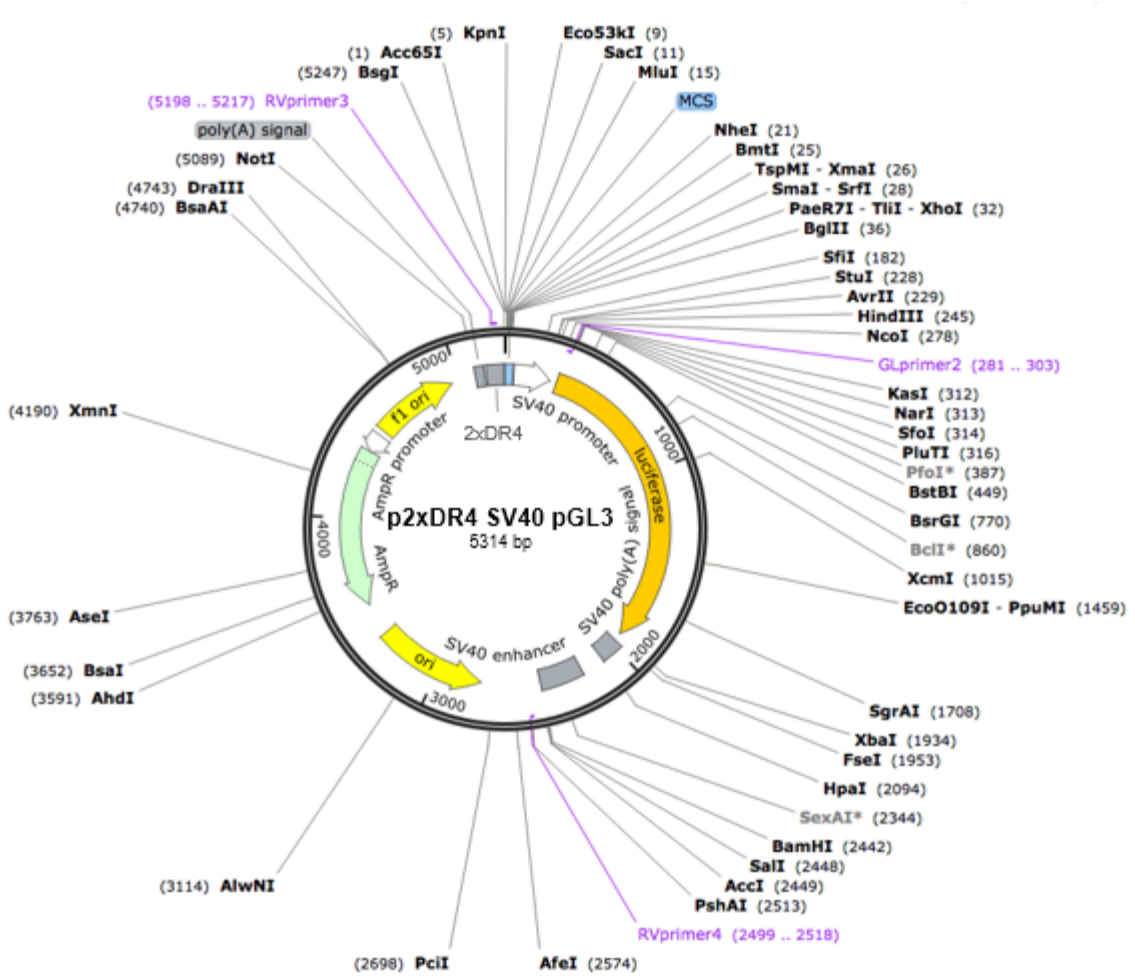

Figure 3: Schematic of pGL3 vector containing 2 DR4 thyroid response elements and SV40 promoter region upstream of a luciferase reporter gene and an SV40 poly(A) tail

Cells were divided into a 24 well plate and transfected using Lipofectamine 2000 (Invitrogen Cat #11669-019) in antibiotic and serum free medium, according to the manufacturer's instructions. 4 wells were transfected with a GFP vector and *Renilla* (transfection control), 10 with the DR4/SV40 vector, *Renilla*, and GFP (positive control), and 10 with the DR4/SV40 vector construct, *Renilla*, and a synthetic TRa1 receptor. Transfected cells were incubated at 37 ºC, 5% CO_2 for a 24-hour period.

Cells were treated in a duplicate fashion in DMEM supplemented with antibiotics. Treatments were re-suspended in a DMSO (Sigma Cat #472301) vehicle. Treatment groups for both the positive control and the experimental group consisted of untreated cells, DMSO, 5 nM T_3 (Sigma-Aldrich Cat #T6397), 10 µM methoxychlor (Cat #), and a 5 nM T_3 and 10 µM mixture.

Luciferase assay

After a 24-hour incubation period, cells were lysed and a dual luciferase assay was performed using the Dual Luciferase Reporter Assay System (Promega Cat #E1910) according to manufacturer's instructions. Luminesence readings were taken in RLU measurements using a Packard LumiCount Luminescence Microplate Reader.

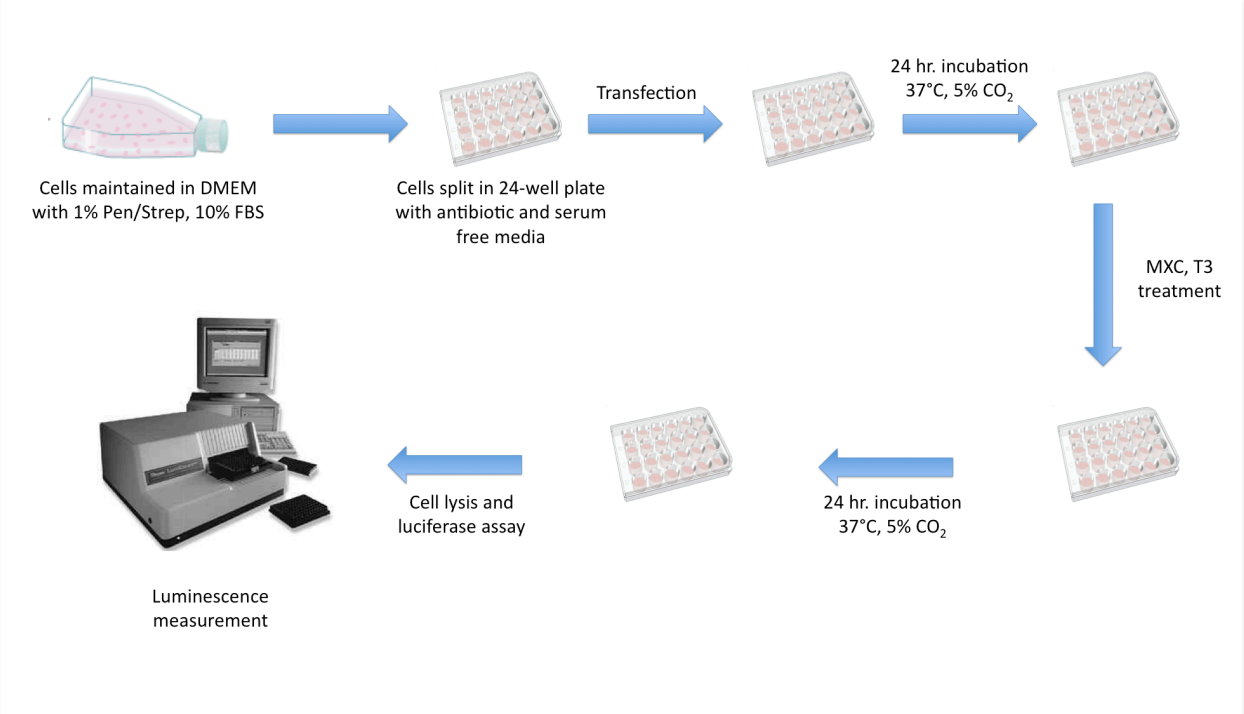

Figure 4: Diagrammatic representation of the procedure that was followed

CHAPTER III

RESULTS

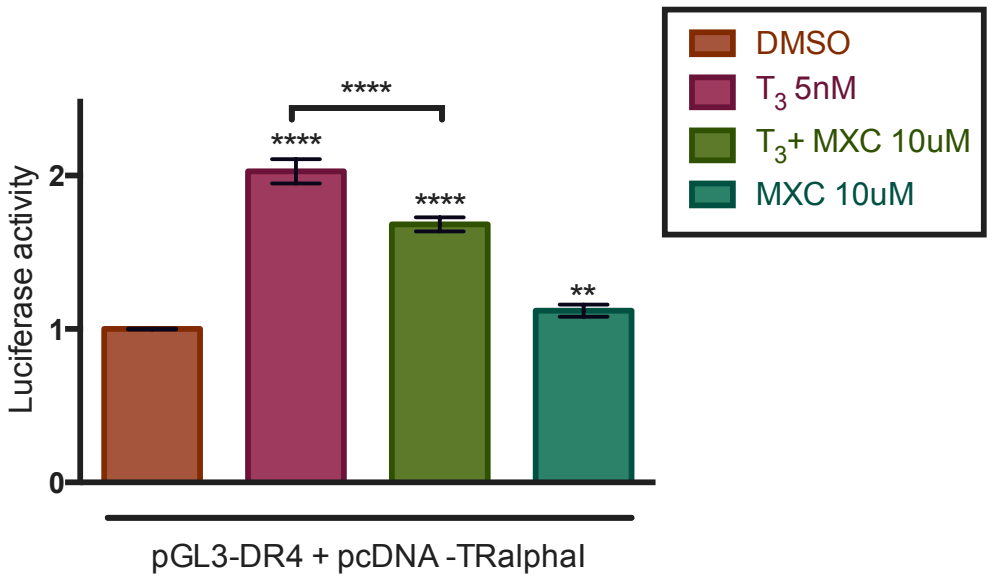

Figure 4: Effects of MXC on T3 regulated transcriptional activity. Luciferase assay using HEK-293 cells transfected with DR4-SV40 luciferase construct and further treated with vehicle (DMSO), 5nM 3,3',5-Triiodo-L-thyronine (T3), 10uM MXC, or T3 and MXC. Statistical significance was determined by an ANOVA followed by a Fischer's exact test (**p < 0.0001,***p < 0.001, **p < .01, relative to vehicle control).**

A dual luciferase assay was performed to assess transcriptional activity. An ANOVA test and a Fischer's exact test were then used to compare transcription levels of T3 treated cells that were further exposed to methoxychlor. The graph above represents the results of six biological replicates.

As shown above, there was an upregulation of transcription when cells were treated with only T3 ($p<0.0001$). Treatment of transfected cells with only methoxychlor also resulted in an increase in transcriptional activity ($p<0.01$). What is of greatest interest, however, is that expression of the luciferase reporter gene decreased in cells that were treated with thyroid hormone as well as methoxychlor ($p<0.0001$).

CHAPTER IV

CONCLUSIONS

Discussion

Because several genes contain thyroid response elements in the enhancer region upstream of the gene, determining how methoxychlor and other environmental toxins affect activity within this region is crucial in understanding their impact on fetal development as a whole.

Cells that were treated with T3 and concurrently exposed to methoxychlor showed a decrease in transcriptional activity. A lower expression of the luciferase reporter gene suggests methoxychlor may interact with thyroid hormone signaling processes, and that genes containing thyroid response elements in their enhancer region are affected by exposure to methoxychlor. The data from this project shows that methoxychlor interferes with thyroid hormone's ability to regulate gene expression.

Future Extensions

There are several steps that can be taken to validate the findings of this project and to understand thyroid hormone's role in regulation of gene expression.

Reverse transcription polymerase chain reaction (RT-PCR) could be utilized to quantitatively detect gene expression. This technique could be used to confirm that the luminescence values

obtained from the luciferase assay were resulting from transcriptional activity of the vector construct. Repetition of the procedure outlined above using a neural stem cell, neural crest cell, or oligodendrocyte cell line would be used to confirm findings in a biologically relevant model (versus the synthetic model utilized in this project). T3-treated cells could also be exposed to a range of doses of methoxychlor in a dose response study to determine a threshold level of exposure before transcriptional activity is affected.

To truly understand how methoxychlor affects thyroid hormone's ability to regulate gene expression during neural development, transcriptional activity should be assessed in cell lines transfected with constructs consisting of genes, such as Sox2, containing thyroid response elements in their enhancer regions. This would allow us to quantitatively determine how an alteration of transcriptional activity in a gene's enhancer region affects downstream expression.

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